

Rescue of a *Wnt* mutation by an activated form of LEF-1: Regulation of maintenance but not initiation of *Brachyury* expression

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Members of the LEF-1/TCF family of transcription factors have been implicated in mediating a nuclear response to Wnt signals by association with β -catenin. Consistent with this view, mice carrying mutations in either the *Wnt3a* gene or in both transcription factor genes *Lef1* and *Tcf1* were previously found to show a similar defect in the formation of paraxial mesoderm in the gastrulating mouse embryo. In addition, mutations in the *Brachyury* gene, a direct transcriptional target of LEF-1, were shown to result in mesodermal defects. However, direct evidence for the role of LEF-1 and *Brachyury* in *Wnt3a* signaling has been limiting. In this study, we genetically examine the function of LEF-1 in the regulation of *Brachyury* expression and in signaling by *Wnt3a*. Analysis of the expression of *Brachyury* in *Lef1*^{-/-}*Tcf1*^{-/-} mice and studies of *Brachyury::lacZ* transgenes containing wild type or mutated LEF-1 binding sites indicate that *Lef1* is dispensable for the initiation, but is required for the maintenance of *Brachyury* expression. We also show that the expression of an activated form of LEF-1, containing the β -catenin activation domain fused to the amino terminus of LEF-1, can rescue a *Wnt3a* mutation. Together, these data provide genetic evidence that *Lef1* mediates the *Wnt3a* signal and regulates the stable maintenance of *Brachyury* expression during gastrulation.

Signaling by Wnt proteins regulates multiple biological processes that include cell fate decisions, cell proliferation, cell polarisation, and morphogenetic movements (1, 2). Genetic analysis of Wnt signaling in the mouse by targeted inactivation of *Wnt* genes has revealed a role of this signaling pathway in the patterning of tissues, such as the isthmus organizer of the midbrain/hindbrain boundary and the formation of organs and tissues, including kidney and placenta (3–6). In addition, a targeted null mutation in *Wnt3a* has been shown to impair the formation of paraxial mesoderm in the early gastrulating mouse embryo (7). *Wnt3a* mutant mice form extra neural tubes at the expense of mesoderm, suggesting that *Wnt3a* regulates the balance between mesodermal and neural fates (8). A naturally occurring hypomorphic mutation of *Wnt3a*, termed *vestigial tail* (*vt*), which is allelic with *Wnt3a*, decreases the level of *Wnt3a* expression and abrogates tail formation in homozygous mice (9). The *vt* mutation also impairs the generation of caudal vertebra, consistent with the mutant phenotype of the *Wnt3a* null mutation.

Biochemical experiments and cell transfection assays have been used to show that the transcriptional effects of signaling by Wnt proteins are mediated by LEF-1/TCF transcription factors (10–15). LEF-1 has no transcriptional activation potential by itself and can stimulate transcription only in collaboration with other proteins (16). In the absence of Wnt signals, LEF-1 can associate either with coactivators, such as ALY (17), or with corepressors, such as Groucho (18), to activate or repress specific target genes, respectively (17, 18). In the presence of Wnt signals, LEF-1/TCF proteins associate with β -catenin and activate transcription from synthetic enhancers containing multimerized LEF-1/TCF binding sites or from natural Wnt-responsive enhancers or promoters in transfected tissue culture cells (11, 13,

19–21). The association of β -catenin with LEF-1/TCF proteins is mediated through evolutionarily conserved amino acids at the amino terminus of the transcription factors (19, 22, 23). β -catenin contains amino- and carboxy-terminal transcriptional activation domains and a covalent fusion of either domain with the amino terminus of LEF-1 generates a fusion protein that functions constitutively and activates transcription independently of a Wnt signal (19).

The number of direct target genes for Wnt signals in the mouse has been limiting. *Brachyury*, one of the earliest genes expressed at the onset of gastrulation during embryonic stage 6.5 (E6.5), has been shown to be regulated by *Wnt3a* signaling in the posterior mesoderm (7, 24, 25). Likewise, the transcription factor genes *Lef1* and *Tcf1* are expressed in the primitive streak during gastrulation in a pattern that overlaps that of *Wnt3a* (26, 27). Although targeted mutation of *Lef1* or *Tcf1* genes alone did not generate any phenotype that resembled that of the *Wnt3a* mutation, inactivation of both *Lef1* and *Tcf1* resulted in a *Wnt3a*^{-/-}-like phenotype (26, 28, 29). Thus, these transcription factors act redundantly to regulate mesoderm formation in the gastrulating mouse embryo, presumably by mediating a nuclear response to a *Wnt3a* signal (26). A role of LEF-1/TCF transcription factors in the regulation of *Brachyury* has been implicated from experiments demonstrating that the *Brachyury* promoter contains binding sites for LEF-1 that are functionally important for expression of a transgene in early mouse embryos (24, 25). Taken together, these experiments suggest that LEF-1 and *Brachyury* form a transcriptional cascade in the transduction of *Wnt3a* signaling in the early mouse embryo. However, no formal genetic proof for the role of LEF-1/TCF proteins in Wnt signaling has yet been obtained.

In this study, we demonstrate that LEF-1 is required specifically for the maintenance, but not the initiation of *Brachyury* expression. In addition, the rescue of the tail deficiency in *Wnt3a*^{vt/vt} embryos with a constitutive β -catenin-LEF-1 fusion protein provides direct genetic evidence that LEF-1 is a downstream effector of signaling by *Wnt3a*.

Materials and Methods

Transgene Construction. The *Xba*I-*Bgl*II fragment from pEVRF0 (30) containing the splice donor, IVS2, and exon 3 with the polyadenylation site from rabbit β globin gene was subcloned into Bluescript II KS+ to generate pBSpA. This gene construct was used for insertion of the murine *Brachyury* promoter (nucleotides -429 to +221 in ref. 31; coordinates are taken from ref. 32). The resulting gene construct, pBrapA, was used for subsequent cloning of the *catC-Lef1* fusion gene or the bacterial *lacZ* gene. The *catC-Lef1* fusion gene, in which the C-terminal

Abbreviations: En, embryonic stage *n*; vt, vestigial tail.

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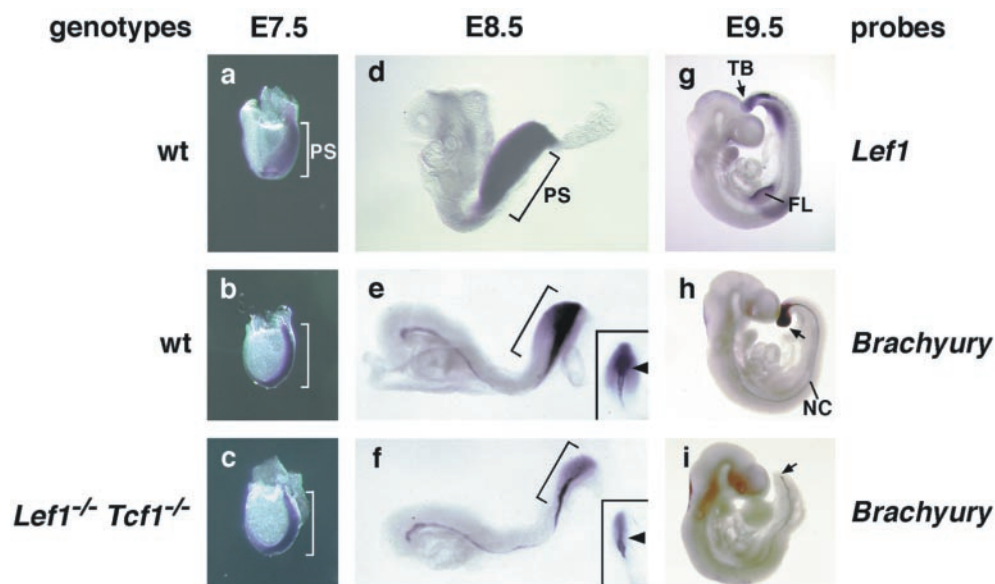


Fig. 1. Maintenance but not initiation of *Brachyury* expression is regulated by LEF-1 and TCF-1 during early mouse development. Whole mount *in situ* hybridizations of wild-type and *Lef1*^{-/-} *Tcf1*^{-/-} embryos between days 7.5 and 9.5 of gestation (E7.5–E9.5) to detect *Brachyury* or *Lef1* transcripts. At E7.5, *Brachyury* expression can be detected in the primitive streak (PS) of both wild-type and compound mutant embryos (b and c). In E8.5 mutant embryos, expression in the primitive streak is reduced relative to the wild-type embryo (e and f). Expression of *Brachyury* is specifically reduced in the anterior part of the primitive streak, similar to *Wnt3a*^{-/-} embryos (Insets in e and f; ref. 24). At E9.5, compound mutant embryos contain *Brachyury* transcripts only in the notochord (NC), whereas wild-type embryos express *Brachyury* in both the tailbud (TB, arrow) and the notochord. For comparison, *Lef1* shows a temporal and spatial expression pattern that overlaps the expression pattern of *Brachyury* in wild-type embryos (a, d, and g). In addition, *Lef1* expression is observed in the forelimbs (FL).

activation domain of human β -catenin (amino acids 696–781) is linked to the amino terminus of LEF-1, was obtained from pCMV-catCLEF-1 (19). The *lacZ* DNA fragment was derived from hsp68lacZpA (33). In the resulting plasmids, pBra-catCLEF1 and pBra-lacZ, the 5' untranslated region and the initial methionine of *Brachyury* were fused in frame to the *catC-Lef1* and *lacZ* genes, respectively. Both gene constructs were sequenced to confirm the reading frame. pmutBra-lacZ was generated by site-directed mutagenesis, using single-stranded DNA from pBra-lacZ and the mutant oligonucleotide 5'-TGCTCGGTACTTGAATTCGTGTCCCGC. DNA constructs were digested with *Sac*II and *Kpn*I, and the inserts were purified to remove vector sequences and used for pronuclear injection following standard protocols (34).

Genotyping and Wnt3a-Vestigial-Tail Analysis. Transgenic animals carrying the wild-type *Bra-lacZ* or mutant *mutBra-lacZ* transgenes were dissected at the indicated times of gestation and the yolk sacs were used for PCR genotyping with primers derived from the *Brachyury* promoter 5'-GAAGTGAAGGTGGCTGT-TGG and the *lacZ* gene 5'-CGATCGGTGCGGGCCTCT-TCGCTAT. To identify animals carrying the *catC-Lef1* transgene, genotyping was performed by PCR using primers for the human β -catenin gene 5'-CGTTCTTTTCACTCTGGTGGA and the murine *Lef1* gene 5'-GTTAACCAAAGATGACTT-GATG. *Lef1*^{-/-} *Tcf1*^{-/-} embryos were generated and genotyped as described (26).

The vestigial tail mouse stock B6C3H-a/A-wa2-vt was purchased from Jackson Laboratory (Bar Harbor, ME) and maintained by backcrossing with C57BL6/J mice. Genotyping was performed by simple sequence length polymorphism (SSLP) analysis as described (9).

Electrophoretic Mobility Shift Assay. DNA binding assays were performed as described (19). The binding reaction contained 100 ng of purified His-6-tagged LEF-1 protein and 2 fmol of 5' end-labeled double-stranded oligonucleotide in 10 mM Hepes

(pH 7.9), 50 mM NaCl, 1 mM DTT, 5 mM EDTA, 5% glycerol, 50 μ g/ml BSA, and 10 μ g/ml poly(dI-dC)-poly(dI-dC). The binding reaction was performed in the absence or presence of increasing amounts of unlabeled competitor DNA. The nucleotide sequences of the probes for *Brachyury* site I (BraSI) and competitor DNA were as follows. Bra-SI wt: 5'-TGCTCGGTACTTCAAAGGGTGTCCCGCTAGCGATC; Bra-SI mut: 5'-TGCTCGGTACTTGAATTCGTGTCCCGCT-AGCGATC; competitor wt: 5'-GCACCCTTTGAAGCTCG-CTAGCGATC; competitor mut: 5'-GCACCAATTCAAGCT-CGCTAGCGATC.

Xgal Staining and Whole Mount *in Situ* Hybridization. Dissected embryos were fixed with 4% paraformaldehyde in PBS for 10 min on ice and stained for β -galactosidase activity by incubation in PBS containing 2 mM MgCl₂, 5 mM K₄Fe(CN)₆, 5 mM K₃Fe(CN)₆, and 0.4 mg/ml X-gal at 37°C for 2 to 24 h. After staining, the embryos were washed in PBS and further fixed in 4% paraformaldehyde in PBS at 4°C. The procedure and the probes for *in situ* hybridizations were the same as described (26).

Results

A role of LEF-1/TCF factors in the regulation of *Brachyury* was inferred from recent experiments in which mutations of binding sites for LEF-1 in the *Brachyury* promoter, linked to a *lacZ* transgene, were found to result in the loss of transgene expression in E9.5 embryos (24). Therefore, the question arose as to whether LEF-1 and TCF-1 regulate the expression of *Brachyury* throughout early mouse development. Toward this end, we examined the expression pattern of *Brachyury* in wild-type and *Lef1*^{-/-} *Tcf1*^{-/-} mice at different stages of development (Fig. 1). As anticipated, *Brachyury* expression could not be detected in compound mutant embryos at E9.5 (Fig. 1i). However, expression of *Brachyury* was detected in *Lef1*^{-/-} *Tcf1*^{-/-} mice at E8.5, albeit at a reduced level relative to wild-type embryos (Fig. 1f). At E7.5, expression of *Brachyury* in compound mutant embryos was almost indistinguishable from that in wild-type embryos

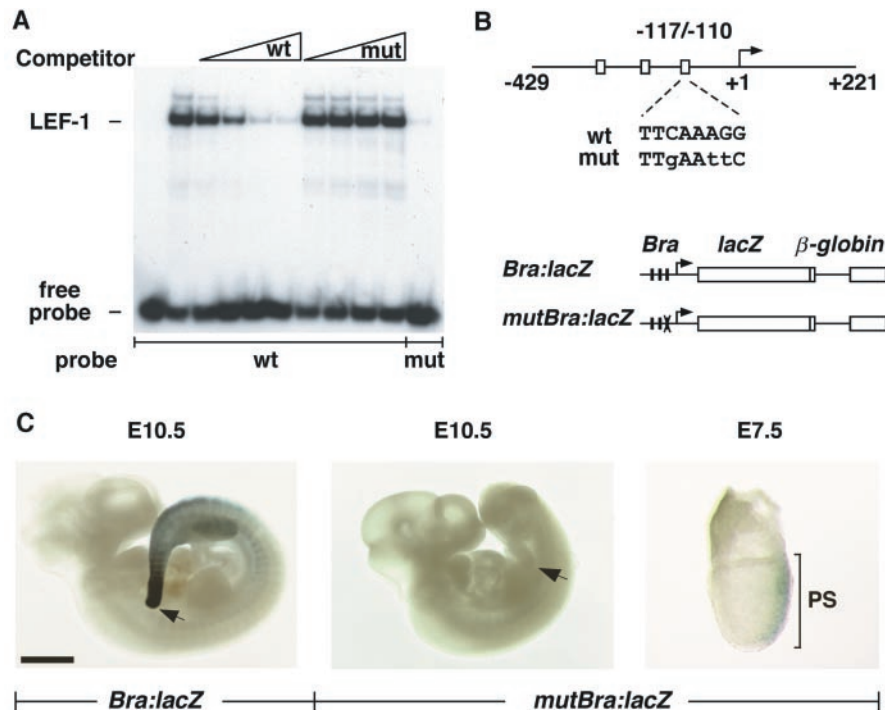


Fig. 2. Brachyury is a direct target of LEF-1. (A) Electrophoretic mobility shift assays to detect binding of purified recombinant LEF-1 to sequences in the *Brachyury* promoter. Specific binding to the promoter-proximal LEF-1 recognition sequence (5'-TTCAAAGG, nucleotides -117/-110) is shown by a competition experiment in which the protein-DNA complex is sensitive to the addition of an excess of a LEF-1 binding site consensus oligonucleotide (5'-TTCAAAGG), but is resistant to the addition of a corresponding mutated oligonucleotide (5'-TTGAATTTC). The positions of the bound and free DNA probes are indicated. (B) Structure of a *Brachyury:lacZ* transgene in which the wild-type or mutated *Brachyury* (*Bra*) promoter is linked to the bacterial β -galactosidase (*lacZ*) gene. To allow for stable transcript accumulation, intron and polyadenylation sequences from the human β -globin gene were included. The nucleotide sequences of the wild-type (wt) and mutated (mut) proximal LEF-1 binding site are indicated. (C) Expression of the *Bra:lacZ* and *mutBra:lacZ* transgenes in E10.5 embryos. Thirteen of 16 transgenic embryos expressed the wild-type transgene in the tailbud (arrow), whereas none of 13 embryos expressed the mutant transgene. In E7.5 embryos, the mutant transgene is expressed in the primitive streak (PS). (Scale bar, 800 μ m for the Left and Center and 225 μ m for the Right.)

(Fig. 1 *b* and *c*). Because other members of the *Lef1/Tcf* family are not expressed at significant levels in the posterior region of the early mouse embryo (26, 35), this result suggests that LEF-1 and TCF-1 act specifically in the maintenance, but not in the initiation of *Brachyury* expression.

To examine whether this temporal regulation of *Brachyury* expression is mediated by direct binding of LEF-1 to the *Brachyury* promoter, we generated transgenes in which the wild-type or a mutated *Brachyury* promoter was fused to the bacterial *lacZ* gene (*Bra:lacZ*). The *Brachyury* promoter contains three binding sites for LEF-1 (24, 25). We introduced a triple point mutation in the proximal LEF-1 binding site (-117/-110) and confirmed by competition in electrophoretic mobility shift assays that mutation of this binding site significantly impaired the interaction with LEF-1 (Fig. 2 *A* and *B*). Analysis of E9.5-E10.5 embryos carrying the wild-type transgene (*Bra:lacZ*) revealed that *lacZ* is expressed in the tailbud of 13 out of 16 different transgenic mice (Fig. 2C *Left*). In contrast, none of 13 embryos carrying the mutant transgene (*mutBra:lacZ*) showed *lacZ* expression (Fig. 2C *Middle*). Thus, mutation of a single LEF-1 binding site abrogates the activity of the *Brachyury* promoter at E10.5.

We also examined whether the *Brachyury* promoter mediates the transient expression of the endogenous *Brachyury* gene in early *Lef1*^{-/-} *Tcf1*^{-/-} embryos. Toward this end, we analyzed the expression of the *mutBra:lacZ* transgene at E7.5. At this early developmental stage, the mutant transgene was expressed, indicating that the *Brachyury* promoter can establish gene expression in a LEF-1-independent manner (Fig. 2C *Right*). Taken together, these data suggest that LEF-1 is dispensable for

activation of the *Brachyury* promoter, but is required for maintenance of *Brachyury* expression during gastrulation.

Two lines of evidence suggest that LEF-1 and TCF-1 mediate the nuclear response to a Wnt3a signal. First, activation of synthetic LEF-1/TCF enhancers or Wnt-responsive enhancers/promoters in tissue culture cells depends on the association of LEF-1/TCF proteins with β -catenin (13, 19). Second, the targeted inactivation of both *Lef1* and *Tcf1* genes results in a mutant phenotype that is very similar to that of the null mutation of the *Wnt3a* gene (7, 26). In particular, the specific down-regulation of *Brachyury* in the anterior streak of *Lef1*^{-/-} *Tcf1*^{-/-} embryos at early somite stages supports the similarity with the phenotype of the *Wnt3a* mutation (Fig. 1 *e* and *f*; ref. 24). To provide direct genetic evidence for the role of LEF-1 in Wnt signaling, we attempted to rescue a mutation in the *Wnt3a* gene by a constitutively active form of LEF-1. For this experiment, we used a covalent fusion of the carboxy-terminal activation domain of β -catenin with the amino terminus of LEF-1, which can activate a synthetic LEF-1 enhancer in the absence of a Wnt signal (19). In addition, the initial expression of *Brachyury* in *Lef1*^{-/-} *Tcf1*^{-/-} embryos enabled us to use the *Brachyury* promoter for the expression of the LEF-1- β -catenin fusion protein (catC-LEF-1) in Wnt3a-deficient embryos. For the rescue, we chose the naturally occurring hypomorphic allele of *Wnt3a*, termed *vestigial tail* (*vt*). In homozygous mice, this mutant allele results in a reduced level of *Wnt3a* expression and a severe defect in tail formation (9).

We introduced the *Brachyury:Lef1- β -catenin* gene (*catC-Lef1*, Fig. 3A) into the mouse germ line and found by whole mount *in situ* hybridization that the transgene is expressed in the primitive

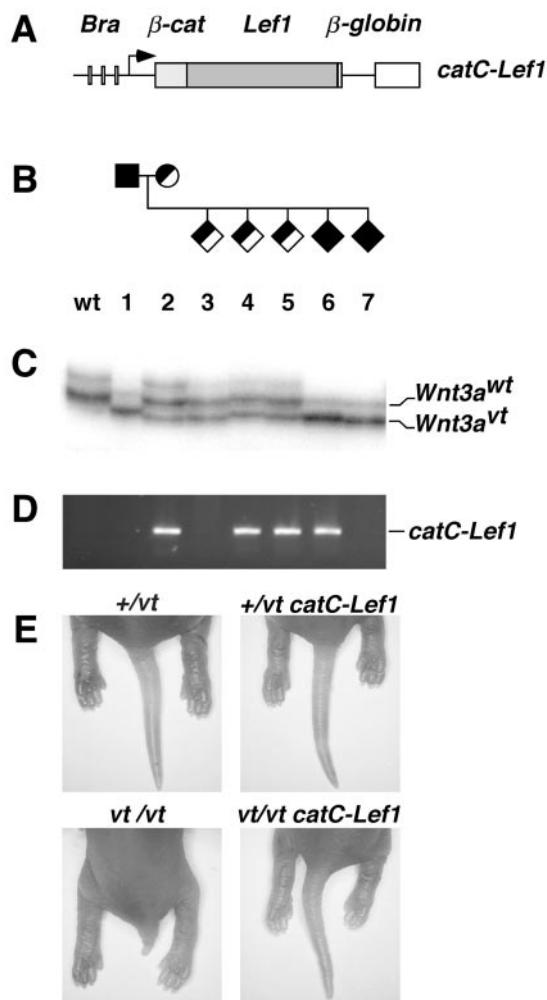


Fig. 3. Rescue of a *Wnt3a* mutation by a β -catenin-LEF-1 fusion protein. (A) Structure of the β -catenin-LEF-1 transgene (*catC-Lef1*), encoding a protein in which the carboxy-terminal activation domain of β -catenin is fused to the amino terminus of full-length LEF-1 (19). The coding sequence is linked to the *Brachyury* promoter [nucleotides -429 to +221 (31, 32) and the rabbit β -globin splice and polyA site (30)]. (B) Pedigree of a cross between a *vt/vt* male (filled square) and *+/vt* female (half-filled circle) carrying the transgene. The offspring are represented by filled (*vt/vt*) or half-filled (*+/vt*) diamonds. (C) Analysis of the wild-type and *vt* allele by simple sequence length polymorphism (SSLP). PCR amplification of *Wnt3a* genomic sequences by using SSLP to detect differences between the wild-type and *vt* alleles (9). (D) Detection of the *catC-Lef1* transgene (Tg) by genomic PCR amplification. (E) Tail phenotypes of newborn littermates from the cross between a *vt/vt* male and a *+/vt* female that carries the *catC-Lef1* transgene. *vt/vt* offspring have a truncated tail, whereas *vt/vt* mice carrying the *catC-Lef1* transgene have a normal tail.

streak of E8.5 embryos (Fig. 4A). To examine whether the *catC-Lef1* transgene could rescue the mutant *Wnt3a*^{vt/vt} phenotype, we crossed the transgene into *vt/vt* mice. A cross between a nontransgenic *vt/vt* male with two *vt/+* females carrying the transgene generated three *vt/vt* offspring carrying the transgene (Fig. 3B and data not shown). The genotyping of the *vt* allele was performed by PCR amplification of a nucleotide sequence polymorphism that is associated with the *vt* allele (9) and the presence of the transgene was determined by genomic PCR (Fig. 3C and D). Newborn *vt/vt* offspring showed the characteristic truncation of the tail, whereas *vt/vt* mice carrying the transgene all had normal tails (Fig. 3E). Wild type and *+/vt* mice carrying the transgene had normal tails (Fig. 3E and data not shown).

This result was confirmed by additional crosses, which produced a total of 50 offspring. From these crosses, only 13 newborns, instead of the expected 25, were tail-less and all of them lacked the transgene (data not shown). Thus, the *catC-Lef1* transgene rescues the defect of the *Wnt3a*^{vt/vt} mutation, providing direct evidence that LEF-1 mediates the *Wnt3a* signal.

To obtain molecular evidence for the rescue of the *Wnt3a*^{vt/vt} mutation by the *catC-Lef1* transgene, we analyzed transgenic and nontransgenic mice for the expression of *Wnt3a*, *Brachyury*, and *Notch1*. We confirmed the proper expression of the transgene by whole mount *in situ* hybridization and compared the expression pattern with that of *Brachyury* at E8.5 and E10.5 (Fig. 4A). A similar pattern of expression was observed, although expression of the transgene was restricted to the more caudal region of the primitive streak and was not detected in the notochord. For the analysis of molecular markers in *Wnt3a*^{vt/vt} and *Wnt3a*^{vt/vt}*catC-Lef1* mice, we used E10.5 embryos because at this developmental stage the expression of endogenous *Wnt3a* is severely reduced in *vt/vt* mice, before the loss of somites can be detected (ref. 9 and Fig. 4).

A positive feedback loop between the target gene of LEF-1, *Brachyury*, and *Wnt3a* has been proposed (24). However, in both *Wnt3a*^{vt/vt} and in *Wnt3a*^{vt/vt}*catC-Lef1* embryos, the expression of *Wnt3a* is markedly reduced (Fig. 4B), indicating that the constitutively active form of LEF-1 does not up-regulate the expression of *Wnt3a*. Expression of *Brachyury*, which is modestly reduced in the tailbud of *Wnt3a*^{vt/vt} mice is normal in *Wnt3a*^{vt/vt}*catC-Lef1* embryos, consistent with the regulation of the *Brachyury* promoter by LEF-1. Thus, these data suggest that *Wnt3a* and *Brachyury* do not form a positive feedback loop. The expression of *Brachyury* in the notochord is also restored in the transgenic mice. Moreover, expression of *Notch1*, a marker for somite formation, is severely impaired in *Wnt3a*^{vt/vt} mice, but is normal in *Wnt3a*^{vt/vt}*catC-Lef1* embryos. Taken together, the expression of the *catC-Lef1* transgene rescues both the morphological and the molecular defects of the *Wnt3a*^{vt/vt} mice, providing formal genetic proof that LEF-1 mediates signaling by *Wnt3a*.

Discussion

One result of our experiments is the finding that LEF-1 and TCF-1 regulate the maintenance, but not the initiation of *Brachyury* expression. A role of Wnt signaling in the maintenance of gene expression has been found in the *Drosophila* embryo, in which Wingless signaling in the epidermis is required for the maintenance of *engrailed* expression (36–38). Likewise, Wnt1 signaling regulates the maintenance of *engrailed-1* expression in the developing central nervous system of the mouse (39). Thus, the *in vivo* analysis of two Wnt target genes in the mouse indicates a role of this signaling pathway in the maintenance rather than initiation of gene expression.

The signals that initiate *Brachyury* expression independently of LEF-1 and TCF-1 are still unknown. Two possible mechanisms may be involved in the initiation of *Brachyury* expression in the primitive streak. Firstly, the *Brachyury* promoter is regulated by FGF and activin signals in *Xenopus* and by activin signals and Ets transcription factors in transfected mammalian P19 cells (32, 40). These factors may be involved in the initiation of *Brachyury* expression during early mouse development. Secondly, *Wnt3* is expressed before gastrulation at E6 in a domain similar to that of the initial *Brachyury* expression (41). In addition, *Brachyury* is not expressed in *Wnt3*^{-/-} embryos, consistent with the initiation of *Brachyury* expression by *Wnt3* (41). This regulation by *Wnt3* could be mediated by the low levels of TCF-3, which is found to be broadly expressed throughout the embryo (35). The observation that the mutant *Brachyury* promoter-lacZ construct is still expressed at E7.5 would argue against this interpretation; however, we mutated only one of three LEF-1/TCF binding sites in the *Brachyury* promoter. Therefore, it is possible that the

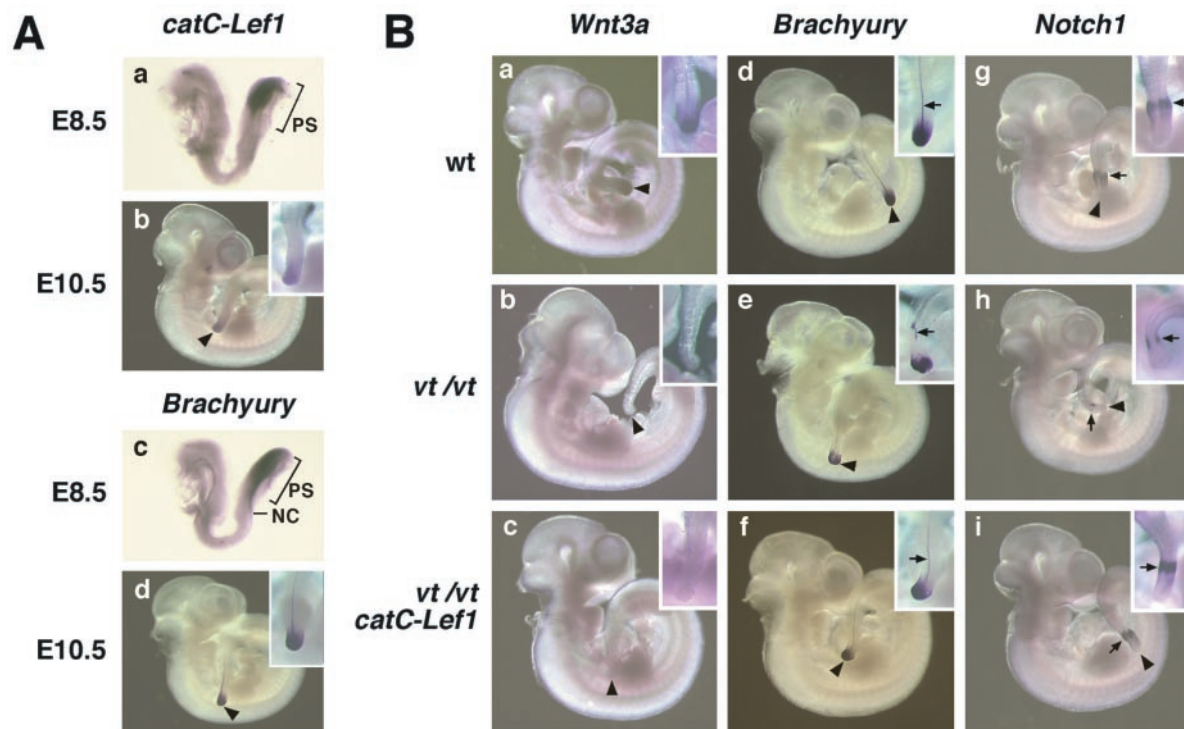


Fig. 4. Molecular analysis of the rescue of the *Wnt3a^{vt/vt}* mutation by expression of a constitutively active form of LEF-1. (A) Whole-mount *in situ* hybridization to detect the expression of the *catC-Lef1* transgene, encoding a protein in which the C-terminal activation domain is fused to the amino terminus of LEF-1, in E8.5 and E10.5 embryos (a and b). Expression of the transgene is detected in the primitive streak (PS) and tailbud (arrow) in a pattern that overlaps with that of endogenous *Brachyury* (c and d). In addition, *Brachyury* is expressed in the notochord (NC). The distal portion of the tails of the E10.5 embryos is shown at a higher magnification in the inset. (B) Expression of *Wnt3a*, *Brachyury*, and *Notch1* in the tailbud of wild-type, *Wnt3a^{vt/vt}* (*vt/vt*), and *Wnt3a^{vt/vt} catC-Lef1* (*vt/vt catC-Lef1*) embryos at E10.5. Whole mount *in situ* hybridization showing that the levels of *Wnt3a* expression in the tailbud (arrow) of a *vt/vt* embryo, which is markedly reduced relative to the wild-type embryo, is also reduced in *vt/vt catC-Lef1* embryos (a, b, and c). In contrast, *Wnt3a* expression in the neural tube is similar in all three embryos. Expression of *Brachyury*, which is slightly reduced in the tailbud of *vt/vt* embryos relative to wild-type embryos, is also affected in the notochord (arrow), presumably because of defective mesoderm formation (d and e). In *vt/vt catC-Lef1* embryos, normal *Brachyury* expression is observed, including a normally formed notochord (f). Expression of *Notch1*, a marker for the presomitic mesoderm in the region adjacent to the first forming somite (45) is reduced in *vt/vt* embryos, but is normal in the *vt/vt catC-Lef1* embryos (g, h, and i). These patterns of expression were confirmed by whole mount *in situ* hybridizations of multiple embryos (data not shown).

remaining two sites mediate the early activation of the promoter. In addition, we cannot rule out the possibility that signaling by Wnt3 is independent of LEF-1/TCF proteins, similar to Wnt5a, which has been found to elicit cellular responses independently of changes in β -catenin levels (42). Although *Wnt5a* is also expressed in the primitive streak, it has been shown that *Wnt5a* is not required for *Brachyury* expression (43). Thus, Wnt3 or FGF signaling might regulate the initiation of *Brachyury* expression, whereas Wnt3a signaling, either alone or in combination with other signaling pathways, controls the maintenance of *Brachyury* expression.

In addition to the potential role of FGF signaling in the initiation of *Brachyury* expression, *Brachyury* has been found to directly regulate the expression of the embryonic *eFgf* gene during mesoderm formation in *Xenopus* (44). The *Xenopus eFgf* gene is closely related to the mammalian *Fgf4* gene, and the regulation of *Fgf4* by *Brachyury* appears to be conserved in mouse and humans (44). Thus, FGF-4 may act both upstream and downstream of *Brachyury*, generating a reinforcing feedback loop that would allow for sustained FGF signaling and differentiation of mesoderm. Although *Wnt3a* itself does not seem to be directly part of this autoregulatory loop, the dependence of *Brachyury* expression on LEF-1 would indirectly link these signaling pathways.

The transcriptional hierarchy of LEF-1 and *Brachyury* allows for temporal regulation of genes, which together with the potential collaboration between different signaling pathways

may help to implement a complex differentiation program. A notable hallmark of LEF-1 is the marked influence of its transcriptional activity on the presence of other specific transcription factors. Although LEF-1 can activate a synthetic enhancer containing multimerized LEF-1 binding sites in transient transfection assays, such a synthetic LEF-1 enhancer is nonfunctional in transgenic *Drosophila* embryos, even in regions in which LEF-1 is coexpressed with wingless, the fly orthologue of Wnt (15). In the *Drosophila ultrabithorax* enhancer, the response to wingless signaling depends on the association of LEF-1 with armadillo and the cooperation of LEF-1/ β -catenin with a member of the CREB family that binds to a dpp response element in the vicinity of a LEF-1 binding site (15). A specific context dependence of LEF-1 function has also been shown for the T cell receptor (TCR) α enhancer, which is regulated by LEF-1 in a β -catenin-independent manner (16). Thus, it will be of interest to compare the LEF-1-mediated regulation of the Wnt3a-responsive *Brachyury* enhancer with that of the β -catenin-independent TCR α enhancer.

Although biochemical experiments and functional studies in tissue culture transfection assays provided strong support for a role of LEF-1 in mediating the response to a Wnt signal, no formal genetic proof has yet been obtained. The rescue of a *Wnt3a* mutation with a constitutively active form of LEF-1 provides now direct evidence for the role of LEF-1 in Wnt3a signaling in the mouse. A similar rescue of a *Wnt1* mutation by an *engrailed* transgene under the control of a *Wnt1* enhancer has

shown that *engrailed* is a critical downstream target of Wnt signaling in formation of the midbrain/hindbrain boundary (39). Similarly, expression of *Brachyury* and *Fgf4* transgenes in Wnt3a-deficient embryos may be used to further examine the transcriptional hierarchy of LEF-1 and Brachyury and to gain insight into the potential network of Wnt3a and FGF signaling in the gastrulating mouse embryo.

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